

Low Temperature Spectral Properties of Subchloroplast Fractions Purified from Spinach¹

Received for publication October 17, 1977 and in revised form December 2, 1977

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ABSTRACT

Spinach (*Spinacia oleracea* L.) chloroplasts solubilized by digitonin were separated into five fractions by sucrose density gradient centrifugation. Three of the fractions, F_I, F_{II}, and F_{III}, corresponding to photosystem I, photosystem II, and the chlorophyll *a/b* complex, were purified further by two steps of diethylaminoethyl-cellulose chromatography followed by electrophoresis on an Ampholine column. The polypeptide patterns of the fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the spectral properties of the fractions at -196°C determined by absorption spectra, fourth derivative curves of the absorption spectra, fluorescence emission spectra, and fluorescence excitation spectra. The activity of purified F_{II} (photosystem II) was also assayed by the photoreduction of dichlorophenol-indophenol at room temperature using 1,5-diphenylcarbohydrazine as the electron donor and by the photoreduction of C-550 at -196°C. The different fractions showed unique polypeptide patterns and unique sets of low temperature-absorbing forms of chlorophyll. The fluorescence emission spectra of F_I, F_{II}, and F_{III} at -196°C were also unique with maxima at 734, 685 and 681 nm, respectively. F_I showed negligible emission at wavelengths shorter than 700 nm and the long wavelength tails of F_{II} and F_{III} in the 730 nm region were relatively small (approximately 10% of emission of their wavelength maxima). Addition of 0.1% Triton to F_I and F_{II} caused the longer wavelength absorbing forms of chlorophyll to shift to 670 nm and the fluorescence emission maxima (of both fractions) to shift to 679 nm at -196°C with an increase in the yield of fluorescence especially in the case of F_I.

The fluorescence emission spectrum of Chl of green plants at -196°C shows emission bands near 685 nm, 695 nm, and 735 nm which have been associated with the three main types of antenna Chl, namely, the light-harvesting Chl *a/b* complex, the antenna Chl *a* associated with PSII units, and the antenna Chl *a* of PSI units, respectively (6). Recently, photochemical models have been developed which permit an analysis of energy distribution in the photosynthetic apparatus, including the yields of fluorescence and photochemistry from PSI and PSII, from measurements of fluorescence at -196°C (6, 8). That analysis, however, depends critically on the fluorescence emission spectra from the three types of Chl which fluoresce at -196°C. To a first approximation the photochemical models assume that the fluorescence measured at 680 nm at -196°C is representative of the fluorescence from the Chl *a/b* complex, that measured at 694 nm at -196°C is representative of the fluorescence from the antenna Chl of PSII, and that measured at 730 nm at -196°C is representative of the fluorescence from the antenna Chl of PSI. The validity of those assumptions depends on the extent to which the emission spectra of the three types of Chl overlap one another. The purpose of the

present work was to extract and purify the three types of Chl complexes and to measure their fluorescence and absorption properties at -196°C. In the course of this work which involved fairly extensive purification procedures, the photochemical properties and polypeptide profiles of the fractions were also examined at different stages of purity.

MATERIALS AND METHODS

The initial purification procedures followed those of Wessels *et al.* (19) and Huzisige *et al.* (10) with some modifications. Chloroplasts which had been prepared by the method of Yamashita and Butler (20) in STN buffer (0.4 M sucrose, 0.05 M Tris-HCl [pH 7.8], 0.01 M NaCl) with 5 mM MgCl₂ and 20 mM sodium ascorbate were centrifuged and resuspended at 3.5 to 4 mg of Chl/ml in 0.05 M Tris-HCl (pH 7.8), with 5 mM MgCl₂ and 1.25% digitonin. After 1 hr at 0°C with stirring, the suspension was centrifuged at 1,000g for 2 min to remove debris and the supernatant centrifuged again at 30,000g for 1 hr at 0°C. The supernatant containing about 30% of the Chl with an *a/b* ratio of 5:8 was discarded and the pellet, now somewhat enriched in PSII, was resuspended in a medium containing 0.05 M Tris-HCl (pH 7.8) with 5 mM MgCl₂, 0.35 M NaCl, and 1.25% digitonin. After 18 to 20 hr at 0°C with stirring, the suspension was centrifuged at 100,000g for 1 hr at 0°C. The pellet was discarded and the supernatant (1- to 1.5-ml aliquots containing about 700 µg of Chl) was placed in each of six centrifuge tubes on a sucrose density gradient, 10 to 30% sucrose in 0.05 M Tris-HCl (pH 7.8), 5 mM MgCl₂, and 0.5% digitonin, and centrifuged 40 to 48 hr at 90,000g at 0°C in an SW 27 rotor. After equilibration had been reached four or five green bands were apparent in the centrifuge tube depending on whether or not the top band could be resolved visually into two bands. Fractions (0.4 ml) were removed from the top of the centrifuge tube with an Instrumentation Specialties Co. model 640 density gradient fractionator which continuously monitored the *A* at 280 nm. Absorption spectra were measured at room temperature and at -196°C on the 0.4-ml fractions. Fractions from a parallel experiment were assayed for PSII activity as the rate of photoreduction of DPIP² with DPC as the electron donor. The P700 content of the fractions was determined from the extent of the light-induced absorbance change at 703 nm at -196°C. The profiles of the various assays made on the fractions from the sucrose density gradient centrifugation are shown in Figure 1.

The fraction, F_{II}, enriched in PSII activity was collected by inserting a syringe needle at the appropriate level on the centrifuge tube and removing 3 to 5 ml, depending on the size of the band. Approximately 25 ml collected from each set of six tubes was placed on a Sephadex G-25 column and eluted with 0.05 M Tris-HCl (pH 7.2) with 0.2% digitonin. The green filtrate was collected and placed on a DEAE-cellulose (DE23) column (2.2 × 3.8 cm)

¹ This work was supported by National Science Foundation Grant PCM 76-07111.

² Abbreviations: DPIP: 2,6-dichlorophenol-indophenol; DPC: 1,5-diphenylcarbohydrazine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

and washed first with 0.05 M Tris-HCl (pH 7.2) and second with 0.075 M Tris-HCl (pH 7.2), both with 0.2% digitonin. The second wash eluted Chl fractions which contained P700 and Chl *b* but no PSII activity. All of the PSII activity was eluted in the next batch elution step made with 0.075 M Tris-HCl (pH 7.2), 0.05 M NaCl, and 0.2% digitonin. This latter fraction was diluted one-tenth with 0.2% digitonin, adsorbed onto a DEAE-cellulose column (1.8 × 2.3 cm) again, washed with 0.075 M Tris-HCl (pH 7.2), and eluted with 0.075 M Tris-HCl (pH 7.2) plus 0.05 M NaCl, both with 0.2% digitonin. Figure 2 shows a gradient elution from DEAE-cellulose. Washing with a 0.075 M Tris-HCl and 0.2% digitonin solution (the first 75 fractions) removed the fraction containing P700 and Chl *b*. The gradient elution with NaCl removed the remainder of the Chl by the time the concentration of NaCl reached 0.05 M. Some carotenoids remained on the column.

The PSII fraction eluted from the DEAE-cellulose was prepared for electrofocusing by dialysis against 5 mM Tris-HCl (pH 7.2) with 0.2% digitonin for 2 hr followed by dialysis against a 0.5% ampholyte solution with 0.2% digitonin for 4 hr. The dialyzed material was then placed in the middle of the Ampholine column (LKB ampholyte 1.5–0.5% from bottom to top) stabilized by a 0 to 50% sucrose density gradient. The electrofocusing on the Ampholine column was run at about 1.2 w for 40 to 45 hr at 2 C. The pH profile of the fractions from the Ampholine column as well as absorbance and fluorescence data are shown in Figure 3. Two colorless protein bands were found at the two ends of the column. The absorbance measurements at 673 nm show that the major Chl *a* band was found at an isoelectric pH of 4.55 with a much smaller band at 4.25 which contained approximately equal amounts of Chl *a* and Chl *b*. This latter band which appeared to be the last vestige of the Chl *a/b* contamination had a very high yield of Chl fluorescence. For measurements of PSII activity the pH of the 4.55 isoelectric point material was adjusted to pH 7.2.

The F_I fraction from the sucrose density gradient separation

was also purified further by DEAE-cellulose column chromatography and electrofocusing on an Ampholine column. A 25-ml sample of F_I collected by syringe from six centrifuge tubes was eluted through a Sephadex G-25 column with 0.01 M Tris-HCl (pH 7.2) with 0.2% digitonin. The green filtrate was adsorbed onto DEAE-cellulose (DE23), washed with 0.01 M Tris-HCl (pH 7.2) with 0.2% digitonin and eluted with 0.075 M Tris-HCl (pH 7.2) with 0.2% digitonin. The DEAE-cellulose step was repeated and the material was prepared for the electrofocusing by the same procedure used for F_{II} . The F_I band focused at an isoelectric pH of about 4.7 on the Ampholine column. A purified F_{III} fraction containing equal amounts of Chl *a* and *b* was also taken off the Ampholine column at an isoelectric pH of 4.25.

PSII activity was measured as the rate of photoreduction of DPIP at 580 nm in a Cary model 17 spectrophotometer. The fractions (2–10 μ g of Chl) were suspended in 1.5-ml samples containing 75 μ mol of Tris-HCl (pH 7.2), 0.05 μ mol of DPIP, 1.5 μ mol of DPC, and 3% ethanol.

Absorption spectra and fluorescence excitation at -196°C were measured with a computer-linked single beam spectrophotometer (4, 11). Fluorescence emission spectra at -196°C were measured from the top surface of the frozen sample with a fiber optics (15). Fluorescence was excited at 633 nm and measured through a

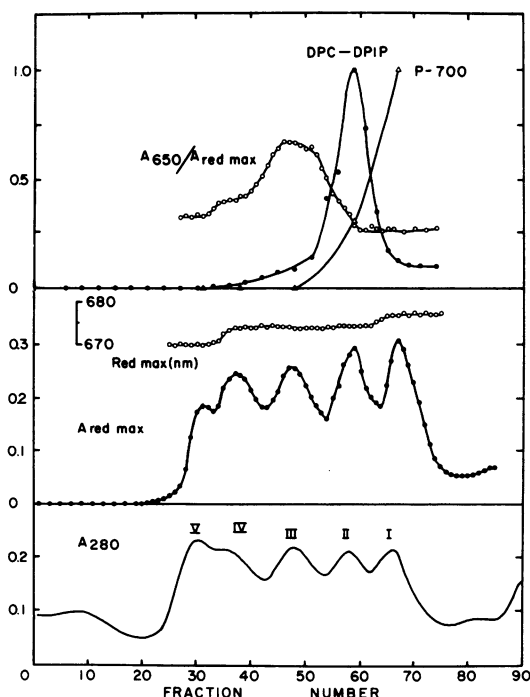


FIG. 1. Fractions obtained from sucrose gradient tube. Bottom panel: elution profile scanned at 280 nm. Middle panel: wavelength of the absorption maximum in the red band (○) and the A at that wavelength (●) both measured at 20°C . Top panel: relative amount of P700 (Δ), relative rate of DPIP photoreduction (●), and the ratio of the A at 650 nm to that of the red maximum at 20°C (○).

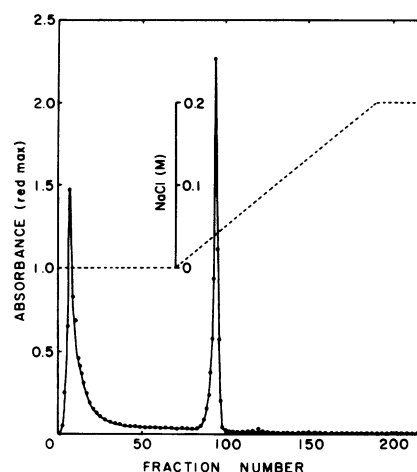


FIG. 2. Gradient elution from DEAE-cellulose column. The column (2.2 × 3.8 cm) was washed with 0.075 M Tris-HCl (pH 7.2) plus 0.2% digitonin, then eluted with the same buffer at increasing NaCl concentration (---). A of 1-ml fractions was measured at the absorption maximum of the red band (—, ●).

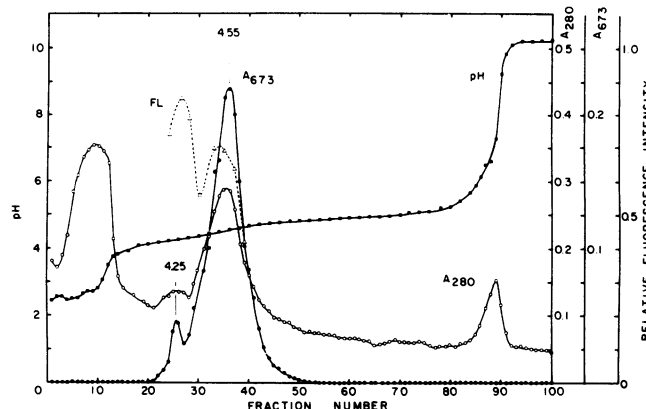


FIG. 3. Fractions obtained from Ampholine column (LKB, 110 ml). pH range of the carrier ampholyte, 4 to 6. For each fraction (1.1 ml), the pH (—) and the A at 280 nm (○) and at 673 nm (●) were measured at 20°C . The relative fluorescence intensities at the emission maximum (681–685 nm) of the fractions (Δ) were measured at -196°C .

monochromator with a 2 nm pass band.

SDS-PAGE was carried out on a slab gel (10% acrylamide) by the method of Laemmli (12). The sample solution containing 0.2 to 0.5 μg of Chl, 10% glycerol, 5% mercaptoethanol, 2.3% SDS (by weight), 0.0625 M Tris-HCl (pH 6.8), and 0.005% bromophenol blue was heated for 1 min at 100 C. The polypeptide bands were stained with 0.05% Coomassie blue, 10% acetic acid, and 25% isopropyl alcohol for 2 hr and destained in 10% acetic acid and 10% isopropyl alcohol overnight. After drying, photographs of the stained slab gels were taken on Polaroid No. 55 P.N film and densitometer tracings were made from the negative film with a microdensitometer (Joyce, Loebel and Co.).

Chl concentrations and Chl *a/b* ratios were determined by the method of Arnon (1). For fractions which contain extremely small amounts of Chl *b* such as the F_{II} fraction from the Ampholine column the Chl *a/b* ratio was determined by the spectrofluorometric method of Boardman and Thorne (2). The relative C-550 content was determined from the light-induced *A* changes at 546 nm measured at -196 C on fractions of known Chl content.

RESULTS

The five components separated by the initial sucrose density centrifugation shown in Figure 1 are labeled sequentially from F_I to F_V starting from the bottom of the centrifuge tube in accordance with the nomenclature of Wessels *et al.* (19). The profiles of the measurements of P700, the photoreduction of DPIP supported by DPC, and the relative *A* at 650 nm on the various fractions establish that F_I, F_{II}, and F_{III} are components highly enriched in PSI, PSII, and the Chl *a/b* protein, respectively, in agreement with the findings of Wessels *et al.* (19).

Absorption and fluorescence emission spectra measured at -196 C on the peak fractions of each of the components are shown in Figure 4 along with the fourth derivative curves of the

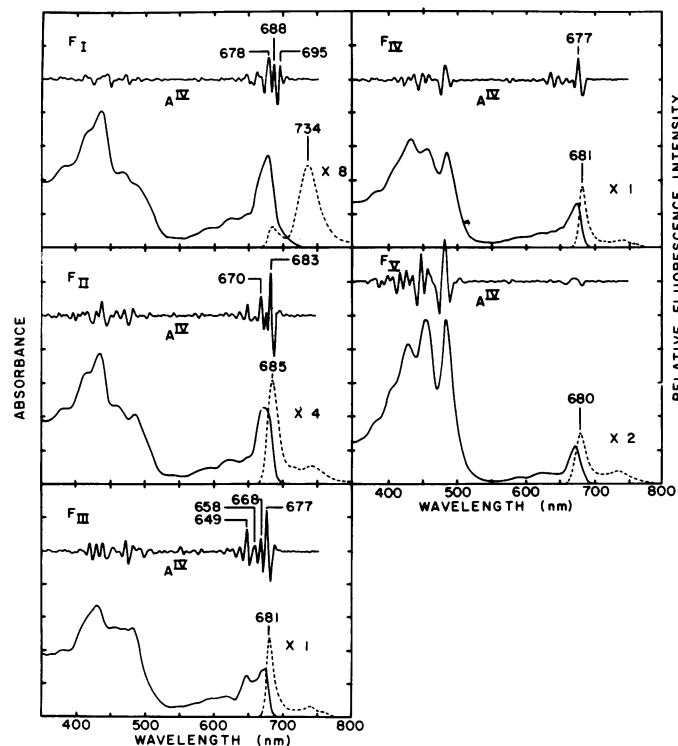


FIG. 4. Absorption and fluorescence emission spectra measured at -196 C on the peak fractions shown in Figure 1. The fourth derivatives of the absorption spectra are shown by curves labeled A^{IV}. Chl concentrations of fractions F_I, F_{II}, F_{III}, F_{IV}, and F_V were 11, 10, 12, 8, and 6 $\mu\text{g}/\text{ml}$, respectively.

absorption spectra. The multiplication factors adjacent to the emission spectra indicate the factor by which the sensitivity was increased for the fluorescence measurements. The Chl concentrations of the various samples were approximately equal so that those factors indicate roughly the relative fluorescence yields of the fractions; *i.e.* the fluorescence yield of F_{III} was about four times that of F_{II} and eight times that of F_I. In general the spectral characteristics of F_I, F_{II}, and F_{III} conform to expectations with almost all of the Chl *b* (649 nm absorption maximum) being associated with F_{III} and with the absorption and fluorescence maxima of F_I being at longer wavelengths than those of F_{II} and both being at longer wavelengths than the maxima of F_{III}.

Most of the carotenoid pigments are associated with F_{IV} and F_V. In order to determine whether the carotenoid pigments were closely associated with the Chl in those fractions, fluorescence excitation spectra were measured at -196 C. Those spectra, shown in Figure 5, indicate that the efficiency of energy transfer from carotenoid pigments in Chl *a* is quite high in F_{IV} but not in F_V. We conclude, therefore, that carotenoid pigments are on the same particles (or the same proteins) as the Chl molecules in F_{IV}. The transfer of energy from carotenoid to Chl suggests that the F_{IV} particles may play a light-harvesting role in the photochemical apparatus but, if so, the details of that interaction are unknown. The shorter wavelength absorption maximum of the red band of F_V at room temperature (*i.e.* 670 nm versus 673 nm for F_{IV}, F_{III}, and F_{II} and 677 nm for F_I, see middle panel of Fig. 1) suggests that Chl in this fraction is less aggregated. F_V may contain "free pigments" which have been separated from the functional particles by the separation procedures.

The fourth derivative spectra in Figure 4 indicate that there is some cross-contamination between the various fractions. For example, the appearance of a band at 649 nm in the fourth derivative spectra of F_I, F_{II}, and F_{IV} indicate that these components are contaminated to some extent with F_{III}. Polypeptide patterns (Fig. 6) obtained from the SDS-PAGE of the various components also indicate some cross-contamination. For example, the doublet near the relative migration position of 4, which is characteristic of F_{II}, is also present in F_{III}. Also, the band characteristic of F_I appears to be present in the patterns of F_{II} and F_{III}.

Most of the contamination of F_{II} with F_I and F_{III} can be removed by the DEAE-cellulose column (Fig. 2) and the small amount of F_{III} which does remain is separated from F_{II} by the electrofocusing procedure (Fig. 3). The electrofocusing also removes significant amounts of nonchlorophyllous protein at the two pH extremes of the column. The SDS-PAGE of F_{II} from the Ampholine column (Fig. 7) confirms that the doublet (bands A and B) at the relative migration position of about 4 is due to F_{II}. Four other polypeptide bands, C, D, E and F, are also a part of

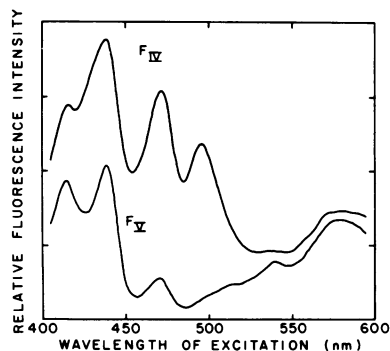


FIG. 5. Fluorescence excitation spectra for peak fractions of F_{IV} and F_V measured at 681 nm at -196 C. The fluorescence was excited with monochromatic light with a 2.8 nm pass band. Chl concentrations for fractions F_{IV} and F_V were 8 and 6 $\mu\text{g}/\text{ml}$, respectively.

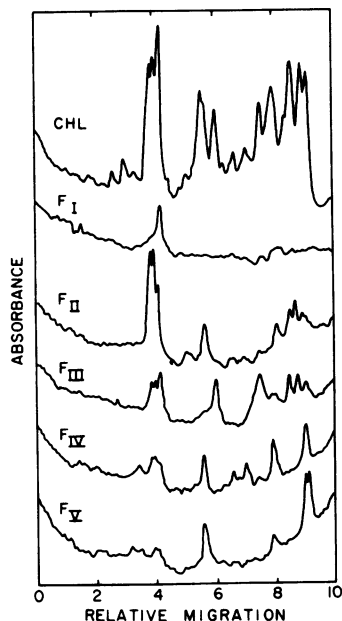


FIG. 6. Densitometric traces of SDS-PAGE of the 100,000g supernatant of digitonin extracts (CHL) and of the five fractions (F_I to F_V) obtained by sucrose gradient centrifugation of the digitonin extract run as described under "Materials and Methods."

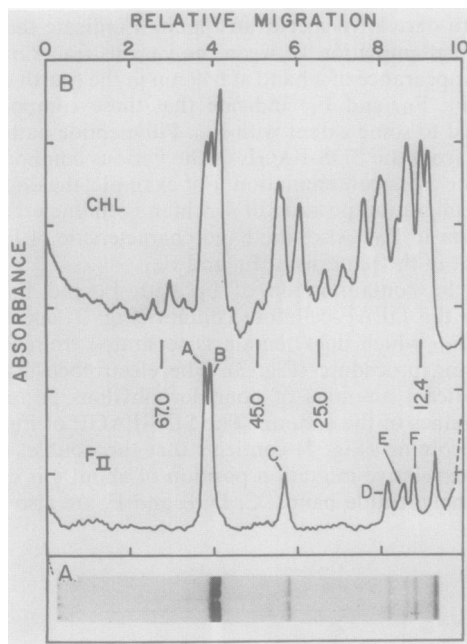


FIG. 7. SDS-PAGE pattern of the purified F_{II} preparations (A) and the densitometric trace (B). Purified samples of bovine albumin (67,000), ovalbumin (45,000), chymotrypsinogen A (25,000), and horse heart Cyt c (12,400) from Mann Research Laboratories used as mol wt markers are indicated at the vertical lines.

F_{II} . The spectral properties of the purified F_{II} at -196°C are shown in Figure 8. The fourth derivative spectrum resolves the main Chl band into two components absorbing at 670 and 683 nm. Some carotenoid pigments are also apparent in the absorption spectrum. The fluorescence excitation spectrum was also measured on the F_{II} fraction at -196°C (Fig. 8B) to determine if both of the absorption bands in the red contributed to fluorescence. Unfortunately this could not be determined for the 685 nm emission band because of its close spectral proximity to the absorption bands so the excitation spectrum was measured for the 750 nm

satellite emission band. The shape of the fluorescence excitation is very similar to the shape of the absorption spectrum. We conclude that 683 nm absorbing form of Chl is responsible for the 685 nm emission band and that the 670 nm absorbing form transfers its excitation energy to the 683 nm form.

The F_I component from the sucrose density gradient centrifugation was also purified further by DEAE-cellulose column chromatography and electrofocusing on the Ampholine column to determine whether the fluorescence of F_I in the 680 nm region in Figure 4 was due to F_I or to contamination, probably by F_{III} . The spectral properties at -196°C of the F_I component after the electrofocusing step are shown in Figure 9. The further purification of F_I , in fact, did remove the 680 nm fluorescence and left a single emission band with a maximum at 734 nm. A small amount of the Chl *a/b* protein was also recovered from the Ampholine column. The fourth derivative spectrum of the purified F_I fraction resolves four absorption bands in the red region at 663, 679, 688, and 695 nm. C-705, which does not appear in the fourth derivative spectrum because of its relatively broad band width, is apparent in the long wavelength tail of the absorption spectrum. C-705 is also apparent in the fluorescent excitation spectrum of the purified F_I fraction measured at 735 nm at -196°C (Fig. 10) as are carotenoid excitation bands.

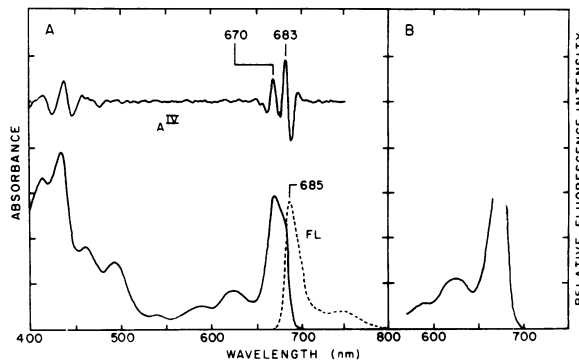


FIG. 8. Absorption and fluorescence emission spectra (A) and fluorescence excitation spectra measured at 750 nm (B) for the purified F_{II} preparations at -196°C . Pass band for fluorescence excitation, 1.1 nm; Chl concentration, 15 $\mu\text{g}/\text{ml}$.

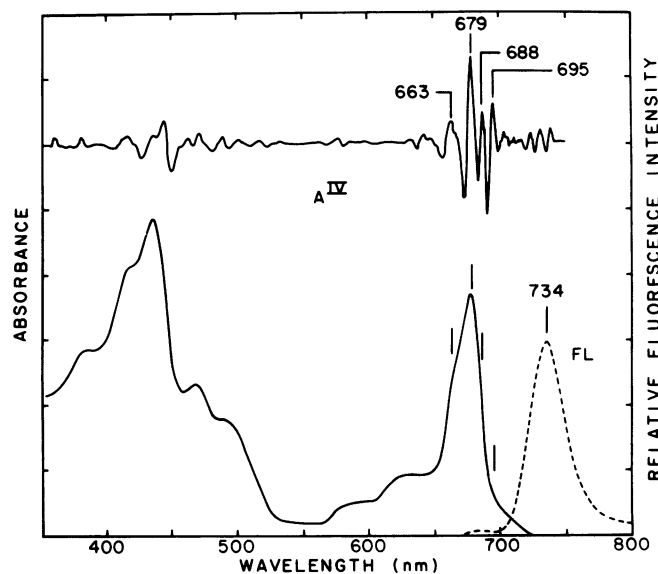


FIG. 9. Absorption and fluorescence emission spectra for the purified F_I preparations at -196°C . Chl concentration, 16 $\mu\text{g}/\text{ml}$.

The fluorescence excitation spectrum of F_I shows an excitation band at about 647 nm which is of interest for questions of energy distribution. The band is not due to Chl *b* since F_I at this stage of purity contains no Chl *b*. The excitation band at 647 nm for PSI fluorescence at -196°C is also apparent in leaves at early stages of greening before any Chl *b* has appeared (18). Thus, both PSI and PSII have excitation bands near 650 nm, that of PSII being due to Chl *b* since the Chl *a/b* complex serves almost entirely as antenna Chl for PSII (8). It has been shown experimentally that the wavelength distribution of energy to PSI and PSII is practically constant from 570 to 680 nm (11). The reason that 650 nm light does not show a strong preference for PSII excitation is that PSI also has an excitation band in the same region as Chl *b*.

The fluorescence emission spectra of F_I , F_{II} , and F_{III} taken from the Ampholine columns are compared in Figure 11. The emission spectrum of F_{III} agrees quite well with that measured previously on the purified Chl *a/b* protein at -196°C (7). The spectra of the purified components also agree reasonably well with the individual spectra obtained from the deconvolution of the fluorescence spectrum of a green leaf at -196°C into its three constituent

components (17). The major discrepancy is the shift of the emission maximum of F_{II} from 694 nm *in vivo* to 685 nm in the purified fraction. The emission spectra in Figure 11 confirm that the emission of F_I is negligible in the 650 to 700 nm region and that the long wavelength emissions of F_{II} and F_{III} in the 730 nm region are quite low. Thus, the emission spectra of the purified components are in agreement with the assumptions made about the emission spectra.

The Purified F_{II} Fraction. The F_{II} fraction from the Ampholine column represents a higher state of purity of PSII than has been obtained previously (19) since the electrofocusing step (Fig. 3) did separate an appreciable amount of protein and some Chl *b* from F_{II} . Some properties of the F_{II} fraction after the different stages of purification are indicated in Table I. The F_{II} fraction from the Ampholine column retained PSII activity with the rate of photo-reduction of DPIP from DPC being about twice that found with chloroplasts on an equal Chl basis. The content of C-550 was markedly enriched in the purified F_{II} particles. The light-induced ΔA changes due to irradiation at -196°C are shown in Figure 12 for a particular F_{II} fraction and for chloroplasts at an equal Chl concentration (2 mm thick sample with $14\ \mu\text{g}$ of Chl/ml). C-550 is scarcely detectable in the dilute suspension of chloroplasts (curve a) but it is clearly apparent in the F_{II} fractions. The midpoint potential of C-550 may have been increased somewhat during the purification since normally C-550 is in the fully oxidized state in the dark. However, with some preparations from some Ampholine columns, the C-550 appeared to be partially reduced since the addition of ferricyanide prior to freezing in-

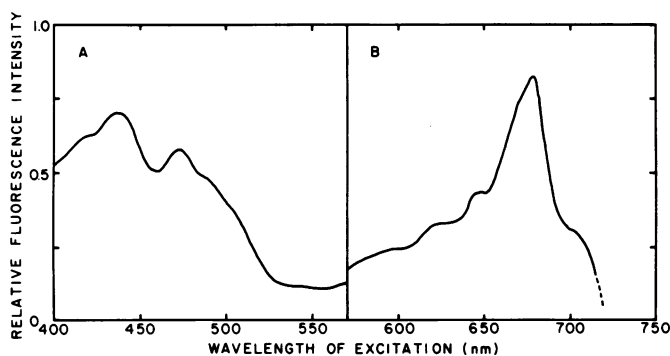


FIG. 10. Fluorescence excitation spectra measured at 735 nm for the purified F_I preparations at -196°C in the blue (A) and the red (B) regions. Pass band for fluorescence excitation, 2.8 nm.

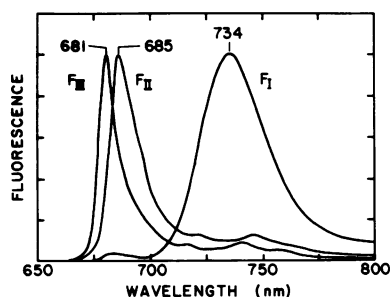


FIG. 11. Comparison of the fluorescence emission spectra of the purified F_I , F_{II} , and F_{III} preparations measured at -196°C . Peak intensities are normalized.

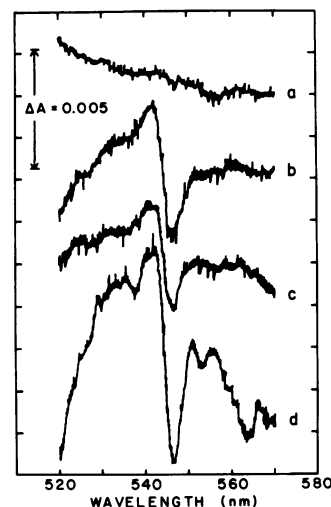


FIG. 12. Light-minus-dark difference spectra of the purified F_{II} preparations at -196°C due to irradiation with white light. a: chloroplasts; b: purified F_{II} ; c: purified F_{II} with 0.5 mM sodium ascorbate; d: purified F_{II} with 0.5 mM potassium ferricyanide. Chl concentrations for all of the samples were $14\ \mu\text{g}/\text{ml}$.

TABLE I. Purification of PSII pigment complex (F_{II}) from spinach chloroplasts

Preparation	Chl		Photochemical activities	
	Total amount	Chl a/Chl b	DPIP reduction by DPC	C-550 content
	mg	ratio	$\mu\text{mol}/\text{mg Chl}\cdot\text{hr}$	relative amount/Chl
Chloroplasts	52.0	3.1	159*	100
Following sucrose gradient centrifugation	4.1	5.5	200	380
Following DEAE column chromatography	2.1	10.0	266	756
Following electrofocusing	1.6	49.0	307	971

*Measured with water as the electron donor

creased the light-induced A due to C-550 (trace d, Fig. 12). We assume that Cyt b_{559} is present in these F_{II} preparations but altered to a low potential form which is not reducible by ascorbate. However, a dithionite-minus-ferricyanide difference spectrum of this sample did show the presence of Cyt b_{559} (data not shown). It is apparent from a comparison of curves a and d in Figure 12 that C-550 is enriched appreciably in the F_{II} fraction.

The mol wt of the six polypeptide bands of F_{II} noted on the SDS-PAGE (Fig. 7) were estimated from a calibration with marker proteins (Fig. 13) to be 55,400, 52,400, 33,700, 17,400, 15,300, and 14,200 (maximum variation $\pm 2,600$). If the area under the bands in Figure 7 is divided by the estimated mol wt, the six components are present at equal ratios (1 ± 0.1). We take this result to indicate that all six are valid functional or structural components of F_{II} .

Effect of Triton. The influence of Triton on the purified frac-

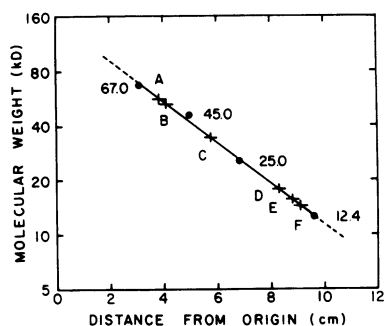


FIG. 13. SDS-PAGE determination of the mol wt of the five polypeptides from the purified F_{II} preparation. See Figure 7 and text for further explanations.

tions was examined because it had been noted earlier (13) that the Chl protein of PSI isolated by Shiozawa *et al.* (14) (CPI) showed a fluorescence emission spectrum at -196°C which was very similar to that of the Chl a/b protein. It appeared that the presence of Triton in the procedures used to obtain CPI caused the emission maximum to shift from 735 nm to about 685 nm. The effect of 0.1% Triton on the spectral properties of the F_I fraction is shown in Figure 14. At -196°C the longer wavelength absorbing forms of the antenna Chl were shifted to shorter wavelength in the presence of the Triton and the emission spectrum was altered from a relatively broad band at 734 nm to a much narrower band at 679 nm with a marked increase in intensity. (The F_I fraction used here was not a peak fraction from the Ampholine column so that there was a small contamination by F_{III} which gives the small fluorescence maximum at 680 nm.) At room temperature where the fluorescence yield of F_I is virtually negligible the addition of the Triton caused a marked increase in the 680 nm emission band.

Similar, though less dramatic, effects were observed on addition of 0.1% Triton to the F_{II} as well (Fig. 15). The 683 nm absorbing form of Chl (seen as the long wavelength shoulder on the absorption spectrum N) was shifted to 670 nm, the fluorescence at -196°C was shifted from 685 to 679 nm, and the intensity of fluorescence at both room temperature and at -196°C was increased by about a factor of 2 by the Triton. We conclude that Triton disengages some of the Chl from the active photochemical apparatus.

DISCUSSION

The emission spectra of the purified fractions F_I , F_{II} , and F_{III} at -196°C support the assumption that the fluorescence emission from each of the three antenna beds of Chl in chloroplasts can be measured separately at -196°C with little overlap from the other two. The emission spectrum of PSII in chloroplasts at -196°C is

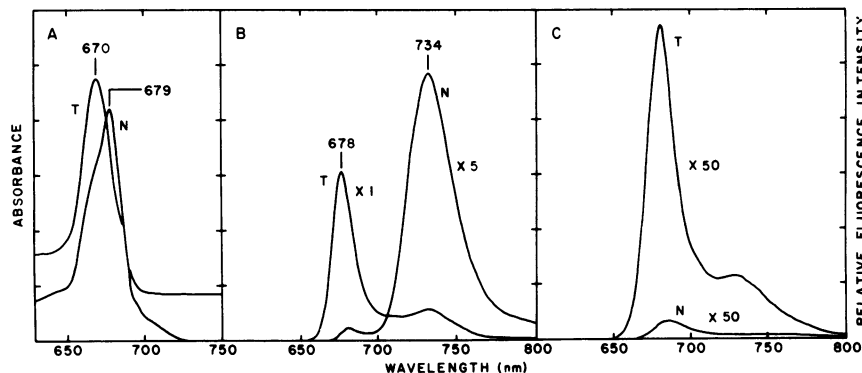


FIG. 14. Absorption spectra measured at -196°C (A) and fluorescence emission spectra measured at -196°C (B) and at 20°C (C) of the purified F_I preparations ($8 \mu\text{g}$ of Chl/ml) in the presence (T) and absence (N) of 0.1% Triton X-100. The multiplication factors adjacent to the emission spectra indicate the factor by which the sensitivity was increased for the measurements.

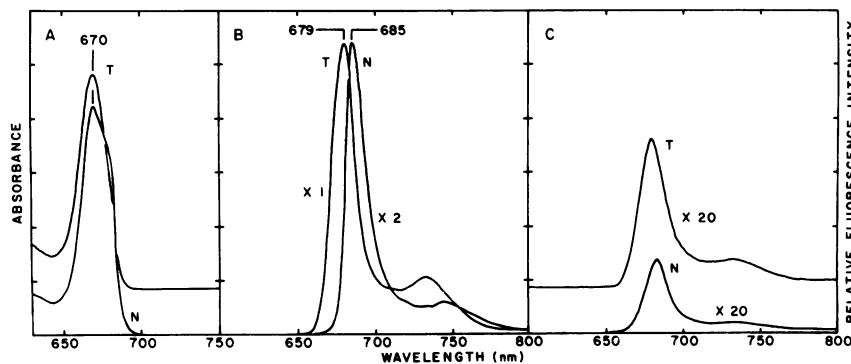


FIG. 15. Absorption spectra measured at -196°C (A) and fluorescence emission spectra measured at -196°C (B) and at 20°C (C) of the purified F_{II} presence (T) and absence (N) of 0.1% Triton X-100.

shifted about 10 nm to longer wavelength from the spectrum of the purified F_{II} fraction which aids the spectral discrimination of F_{II} and F_{III} *in vivo*.

The fourth derivative spectra of F_I , F_{II} , and F_{III} at -196°C show characteristic absorption maxima which can be correlated with similar maxima observed in the fourth derivative spectra of chloroplasts at -196°C (5). The digitonin treatment used to separate the various fractions did not alter the absorption bands to any appreciable extent. However, that would not have been the case if Triton had been present in the separation procedures.

The similarity between the fluorescence excitation spectra and the absorption spectra of F_I (compare Figs. 9 and 10) and of F_{II} (Fig. 8) indicates that all of the pigments are fixed to the particles or protein aggregates and that there is an efficient transfer of energy from the shorter wavelength forms to the longest wavelength pigment which emits the fluorescence. Each particle in the fraction contains the complement of pigments which is characteristic of that fraction. We conclude that Triton disengages some of the antenna Chl molecules from the active photochemical apparatus.

The 735 nm fluorescence of chloroplasts at -196°C has been attributed to a long wavelength form of Chl, C-705, which traps excitation energy from the antenna Chl system of PSI (3). It is apparent from a comparison of the fluorescence excitation spectrum of the F_I fraction (Fig. 10) with the low temperature absorption spectrum of that fraction (Fig. 9) that the direct excitation of C-705 excites the 735 nm fluorescence more efficiently than the indirect excitation via energy transfer from the antenna Chl. That same relationship was noted previously in studies of energy transfer in spinach chloroplasts (16) and in flashed bean leaves (18) at -196°C . We propose that C-705 and P700 compete for the energy in the antenna Chl of PSI. For wavelengths shorter than 700 nm absorbed predominantly by the antenna Chl, the excitation is divided between the two trapping species; at wavelengths longer than 700 nm, C-705 becomes the predominant absorber (at -196°C) so that the yield of fluorescence at 735 nm increases in this long wavelength region. We also assume that C-705 only forms on cooling to low temperature and that the temperature dependence of the 735 nm fluorescence band reflects the temperature dependence for the formation of C-705.

Acknowledgments—We acknowledge many stimulating and helpful discussions with R. J. Strasser.

LITERATURE CITED

1. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol* 24: 1-15
2. BOARDMAN NK, SW THORNE 1971 Sensitive fluorescence method for the determination of chlorophyll *a*/chlorophyll *b* ratios. *Biochim Biophys Acta* 253: 222-231
3. BUTLER WL 1961 A far-red absorbing form of chlorophyll *in vivo*. *Arch Biochem Biophys* 93: 413-422
4. BUTLER WL 1972 Absorption spectroscopy of biological materials. *Methods Enzymol* 24: 3-25
5. BUTLER WL, DW HOPKINS 1970 An analysis of fourth derivative spectra. *Photochem Photobiol* 12: 451-456
6. BUTLER WL, M KITAJIMA 1975 A tripartite model for chloroplast fluorescence. In M Avron, ed. *Proc Third Int Congr on Photosynthesis*. Rehovot, Israel: Elsevier, Amsterdam, pp. 13-24
7. BUTLER WL, M KITAJIMA 1975 Energy transfer between photosystem II and photosystem I in chloroplasts. *Biochim Biophys Acta* 396: 72-85
8. BUTLER WL, RJ STRASSER 1977 Tripartite model for the photochemical apparatus of green plant photosynthesis. *Proc Nat Acad Sci USA* 74: 3382-3385
9. BUTLER WL, RJ STRASSER 1978 Effect of divalent cations on energy coupling between the light-harvesting chlorophyll *a/b* complex and photosystem II. *Proc 4th Int Congr on Photosynthesis*. Reading, England. In press
10. HUZISIGL H, H USIYAMA, T KIKUTI, T ASI 1969 Purification and properties of the photoactive particle corresponding to photosystem II. *Plant Cell Physiol* 10: 441-455
11. KITAJIMA M, WL BUTLER 1975 Excitation spectra for photosystem I and photosystem II in chloroplasts and the spectral characteristics of the distribution of quanta between the two photosystems. *Biochim Biophys Acta* 408: 297-305
12. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685
13. SATOH K, STRASSER R, WL BUTLER 1976 A demonstration of energy transfer from photosystem II to photosystem I in chloroplasts. *Biochim Biophys Acta* 440: 337-345
14. SHIOZAWA JA, RS ALBERTL, JP THORNBLE 1974 The P700 chlorophyll *a* protein. Isolation and some characteristics of the complex in higher plants. *Arch Biochem Biophys* 165: 388-397
15. STRASSER RJ, WL BUTLER 1976 Energy transfer in the photochemical apparatus of flashed bean leaves. *Biochim Biophys Acta* 449: 412-419
16. STRASSER RJ, WL BUTLER 1977 Energy transfer and the distribution of excitation energy in the photochemical apparatus of spinach chloroplasts. *Biochim Biophys Acta* 460: 230-238
17. STRASSER RJ, WL BUTLER 1977. Fluorescence emission spectra of photosystem I, photosystem II and the light-harvesting *a/b* complex of higher plants. *Biochim Biophys Acta* 462: 307-313
18. STRASSER RJ, WL BUTLER 1977 The yield of energy transfer and the spectral distribution of excitation energy in the photochemical apparatus of flashed bean leaves. *Biochim Biophys Acta* 462: 295-306
19. WESSELS JSC, O VAN ALPHEN-VAN WAVEREN, O VOORN 1973 Isolation and properties of particles containing the reaction center complex of photosystem II from spinach chloroplasts. *Biochim Biophys Acta* 292: 741-752
20. YAMASHITA T, WL BUTLER 1968 Inhibition of chloroplasts by UV irradiation and heat treatment. *Plant Physiol* 43: 2037-2040